Arugosins G and H: Prenylated Polyketides from the Marine-Derived Fungus *Emericella nidulans* var. *acristata*

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The fungus *Emericella nidulans* var. *acristata* was isolated as an endophyte from a Mediterranean green alga. Cultivation of this fungus yielded two new compounds, arugosins G (**1**) and H (**2**), together with the known metabolites **3**−**9**. Arugosins (**1**−**4**) are benzophenone derivatives, biosynthetically related to the xanthones **5**, **6**, and **9**. The indole alkaloid **7** displayed antitumor activity in a panel of 36 human tumor cell lines, exhibiting a mean IC_{50} value of 5.5 μg/mL in an in vitro survival and proliferation assay. Furthermore, compounds **3** and **4** showed moderate antitumor activity toward individual tumor cell lines. None of compounds **1**−**8** exhibited any immunostimulatory activity assessed as the capacity to induce cytokines in PBMCs from healthy donors.

Fungi are an extremely valuable source of novel natural products with a wide array of biological activities.[1] Several fungal metabolites, e.g., fumagillin and illudin S,[2] are undergoing clinical trials as antitumor compounds and inspired the current study, which focuses on marine-derived fungi.

As known for higher plants, numerous endophytic fungi are present in marine algae, residing inside the algal tissue. The isolation of fungal endophytes from algae can be easily performed by placing algal thalli, after surface sterilization to remove unwanted epibionts, on suitable agar media. Interestingly, most of the isolated endophytic fungi are not obligate marine fungi. However, they are a tremendous source of natural products, which is not surprising considering the complex ecological situation of the endophyte within the host plant.[1d]

Emericella nidulans var. *acristata* was isolated from a green alga collected around Sardinia in the Mediterranean Sea. HPLC-MS and HPLC-DAD investigations of various extracts identified this fungus as a producer of several polyketide type metabolites. The crude extract also showed cytotoxicity toward six cultured tumor cell lines with a mean IC_{70} of 8.30 μg/mL. Two new compounds from the arugosin family, arugosins G (**1**) and H (**2**), were isolated together with the known arugosins A and B (**3** and **4**). Apart from arugosins, which possess a benzophenone skeleton, and the biosynthetically related (Scheme 1) xanthones **5**, **6**, and **9**, the indole alkaloid **7** and the furanone **8** were obtained. A prominent feature of most of these metabolites is their substitution with prenyl moieties, with the isoprene units being either attached to a carbon atom of the polyketide nucleus or connected via an ether bridge.The fungus *Emericella nidulans* var. *acristata* was cultivated on a solid biomalt medium with added artificial sea salt. Successive fractionation of the EtOAc extract by normal-phase vacuum liquid chromatography (VLC), followed by separation over Sephadex, and normal- and reversed-phase HPLC yielded two new (**1** and **2**) and seven known (**3**−**9**) compounds. Arugosins A and B (**3** and **4**) were isolated as a mixture, as in all previous investigations.[3] The structural elucidation of compounds **1** and **2** is based on NMR and MS data and on comparison of data with those of the known compounds **3** and **4**.

Results and Discussion

The molecular formula of compound **1** was established by high-resolution mass measurement (HREIMS) as $C_{30}H_{36}O_6$, implying 13 degrees of unsaturation. The ^{13}C NMR spectrum showed 30 signals for 7 × CH_3, 3 × CH_2, 6 × CH, and 14 × C. These data also revealed the presence of 10 double bonds (1 × CO, 9 × C=C) (Table 1). Thus, compound **1** was tricyclic. Considering the molecular formula and the IR data (ν_{max} 3438 cm^{-1}), it was obvious that three protons had to be present as hydroxyl groups. UV maxima at 305 and 366 nm pointed toward an extended aromatic moiety. This was supported by two singlet resonances in the ^1H NMR spectrum at δ 7.26 and 6.89 for two aromatic protons. An ^1H NMR resonance at δ 7.03 could be attributed to H-11, which is attached to a doubly oxygenated carbon resonating at 92.2 ppm, as evident from the ^{13}C NMR and HSQC spectra. These structural features suggested compound **1** to be an arugosin derivative, likely to be closely related to arugosins A (**3**) and B (**4**). The major differences between the NMR data for **3** and **4** and **1** were the presence of signals for nine additional protons (δ_H 1.70, 3.29, 5.30; H-1‴ to H-5‴) and five further carbons (δ_C 17.8, 25.8, 28.4, 123.4, 132.5; C-1‴ to C-5‴) in the case of **1**.

The $^1H-^1H$ COSY correlation (Table 1) between H-2‴ and H_2-1‴, and $^1H-^{13}C$ HMBC correlations between H_2-1‴ and C-2‴ and C-3‴ and between both H_3-4‴ and H_3-5‴ and C-2‴ and C-3‴, suggested compound **1** to contain a third 3-methylbut-2-enyl group, which was corroborated by the mass difference between **1** and **3** or **4**. The position of this additional group in compound **1** was deduced from HMBC correlations between H_2-1‴ and both C-4a and C-3. Thus, it was evident that the 3-methylbut-2-enyl group replaced the aromatic proton H-4 of compound **3**, a deduction supported by ^1H NMR data, which showed the absence of the signal at δ_H 6.44 (H-4 in **3**).[3b] All other spin systems deduced from the $^1H-^1H$ COSY and HMBC correlations were consistent with the proposed structure for **1** (Figure 1). Thus, the new compound **1** is the 4-(3-methylbut-2-enyl) derivative of **3**. Due to the similarity between compounds **1** and **3** or **4**, we propose the name arugosin G. As already stated by Gloer et al. for arugosin F,[4] arugosin G also has a small negative optical rotation. Due to the small amount of compound isolated, we did not determine whether the hemiacetal function of arugosin G gives rise to an enantiomeric mixture. The stereochemistry of arugosins A−F has not been reported.

The molecular formula of compound **2** was found to be $C_{20}H_{20}O_6$, as deduced from HREIMS and NMR data, implying 11 degrees of unsaturation. The ^{13}C NMR spectrum showed 20 carbon signals attributable to 3 × CH_3, 1 × CH_2, 5 × CH, and 11 × C (Table 2).

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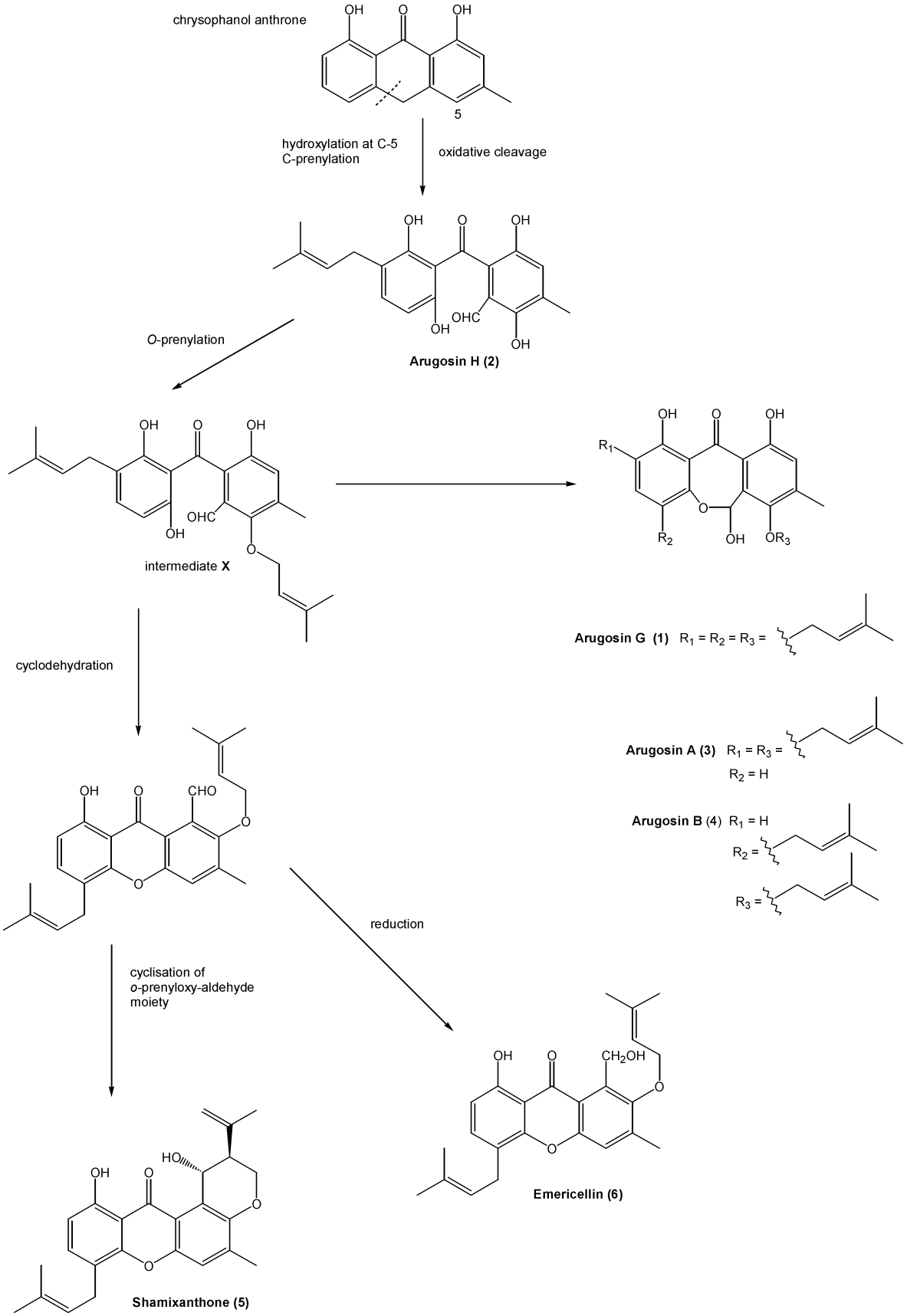
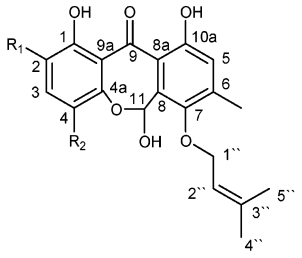
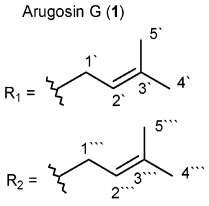
Scheme 1. Proposed Biosynthetic Relationship of *Emericella nidulans* var. *acristata* Secondary Metabolites



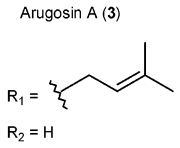
Considering the molecular formula and according to the IR data (ν_{max} 3237 cm^{-1}), it was evident that the four remaining protons had to be part of the hydroxyl groups. The ^{1}H and ^{13}C NMR chemical shifts and HSQC spectra suggested the presence of one ketone (δ_C 200.4) and one aldehyde carbonyl (δ_C 196.2, δ_H 9.86) group, a deduction supported by IR absorptions at ν_{max} 1698 and 1615 cm^{-1}, and seven C═C double bonds. These data, together with the number of unsaturations, required two rings within the molecule, both of which were aromatic. The ^{1}H NMR data showed three aromatic protons, two of them (H-3 and H-4) with an ortho

Chart 1

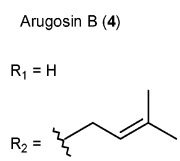




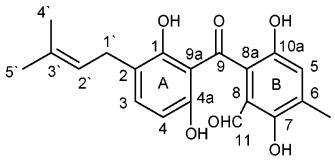
Arugosin G (**1**)



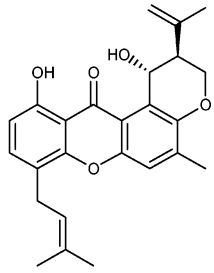
Arugosin A (**3**)



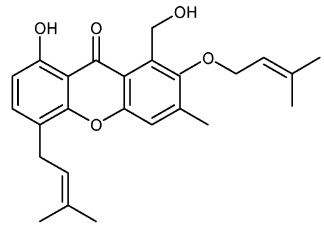
Arugosin B (**4**)



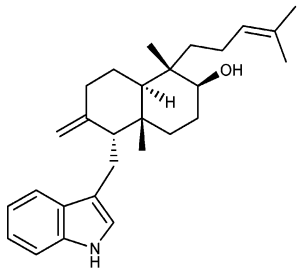
Arugosin H (**2**)



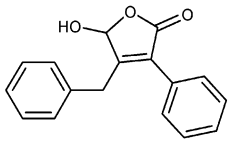
Shamixanthone (**5**)



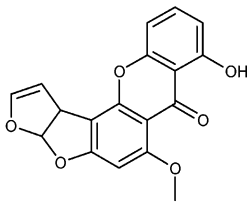
Emericellin (**6**)



Emindole DA (**7**)



Microperfuranone (**8**)



Sterigmatocystin (**9**)

coupling and the third one (H-5) as a singlet. The ^{1}H NMR spectrum also contained a resonance for an aryl methyl group (δ 2.07, H_3-6-Me) and two hydrogen-bonded phenolic OH groups (δ 12.84 and 11.30). The location of the carbonyl group (C-9), as a link between the two aromatic rings at positions C-8a and C-9a, was supported by weak HMBC correlations from both H-4 and H-5 to C-9. The ^{1}H NMR signals observed at δ 1.69 (CH_3-4′ and CH_3-5′), 3.24 (CH_2-1′), and 5.29 (CH-2′) were assigned to a 3-methylbut-2-enyl group, according to the ^{1}H−^{1}H COSY correlations between H_2-1′ and H-2′ and HMBC correlations from H_2-1′, H_3-4′, and H_3-5′ to C-3′. The position of the 3-methylbut-2-enyl group was proven to be at C-2 by correlations in the ^{1}H−^{1}H COSY spectrum between H-3 and H_2-1′ and in the HMBC spectrum between H_2-1′ and C-1, C-2, and C-3. Carbon C-4a had to be hydroxylated due to its resonance in the ^{13}C NMR spectrum (δ 159.4). An ortho coupling between H-3 and H-4 and HMBC correlations between H-4 and C-2, C-4a (weak), C-9 (weak), and C-9a and between H-3 and C-4a and C-1 confirmed the substitution pattern of the aromatic ring A. The remaining two hydroxyls, the aldehyde, and the methyl group resided at the aromatic ring B. Correlations from the ^{1}H−^{1}H COSY between H-5 and H_3-6Me and HMBC correlations from H_3-6Me to C-5 and C-7 and from H-5 to C-7, C-8a, C-9 (weak), and C-10a (weak) indicated the position of the methyl group at C-6 and the positions of the carbons C-5 and C-7 in the aromatic ring B. Hydroxylation of C-7 was suggested by its ^{13}C NMR chemical shift at δ_C 154.5. Although, the proton of the aldehyde group showed no coupling with any of the neighboring carbons, its position was assigned to C-8 on the basis of C-8 being the only remaining nonprotonated carbon. These results are consistent with a bicyclic arugosin structure with the middle ring open. This deduction is supported by biosynthetic considerations (Scheme 1).[5] For compound **2**, the name arugosin H is proposed.

The structures of arugosins A and B (**3** and **4**),[6] shamixanthone (**5**),[5] emericellin (**6**),[3b] emindole DA (**7**),[7] microperfuranone (**8**),[8] and sterigmatocystin (**9**)[9] were identified by comparing their spectroscopic data and optical rotations with published values.

Fungi of the genus *Emericella* (anamorph: *Aspergillus*) produce a great diversity of secondary metabolites.[10] Among them is a family of compounds called arugosins, which are of interest with regard to the biosynthesis of several structural types of fungal polyketides (e.g., anthrones, anthraquinones, benzophenones, xanthones).[5,11] Arugosins A and B, two substituted dibenz[*b*,*e*]oxepins, are the

Table 1. 1D and 2D NMR Spectral Data for Compound **1**

atom no.	$^{13}C^{a,b}$ (δ in ppm)	$^{1}H^{a,b}$ (δ ppm, mult., J in Hz)	$^{1}H-^{1}H$ COSY^{a,b}	HMBC^{a,c}
1	160.5 (C)			
2	122.9 (C)			
3	138.8 (CH)	7.26 (s)	1′,[d] 1‴[d]	1, 4a, 9a,[d] 1′, 1‴
4	122.9 (C)			
4a	150.0 (C)			
5	120.9 (CH)	6.89 (s)	6-Me	7, 8,[d] 8a, 9,[d] 10a
6	141.8 (C)			
6-Me	16.9 (CH_3)	2.35 (s)		5, 6, 7, 8[d]
7	146.3 (C)			
8	133.2 (C)			
8a	119.6 (C)			
9	199.4 (C)			
9a	114.9 (C)			
10a	157.6 (C)			
11	92.2 (CH)	7.03 (s)		7, 8a, 10a[d]
1′	29.4 (CH_2)	3.24 (brd, 7.32)	2′, 3	1, 3, 2′, 3′
2′	123.8 (CH)	5.23 (t, 7.32)	1′	1′
3′	132.8 (C)			
4′	25.8 (CH_3)	1.67 (s)		2′, 3′, 5′
5′	17.8 (CH_3)	1.70 (brs)		2′, 3′, 4′
1″	72.2 (CH_2)	4.42 (d, 7.32)	2″	2″, 3″, 7
2″	120.8 (CH)	5.59 (t, 7.32)	1″	1″
3″	138.9 (C)			
4″	25.8 (CH_3)	1.78 (s)		2″, 3″, 5″
5″	18.0 (CH_3)	1.69 (s)		2″, 3″, 4″
1‴	28.4 (CH_2)	3.29 (d, 7.32)	2‴, 3	3, 4a,[d] 2‴, 3‴
2‴	123.4 (CH)	5.30 (t, 7.32)	1‴	1‴
3‴	132.5 (C)			
4‴	25.8 (CH_3)	1.70 (brs)		2‴, 3‴, 5‴
5‴	17.8 (CH_3)	1.70 (brs)		2‴, 3‴, 4‴
1-OH		13.21 (s)	3[d]	1, 2, 3,[d] 9,[d] 9a
10a-OH		10.59 (s)		5, 6,[d] 8a, 10a
11-OH		6.73 (brs)		8[d]

[a] Acetone-d_6, 300/75.5 MHz. [b] Assignments are based on extensive 1D and 2D NMR measurements (HMBC, HSQC, COSY). [c] Numbers refer to carbon resonances. [d] Weak signal.

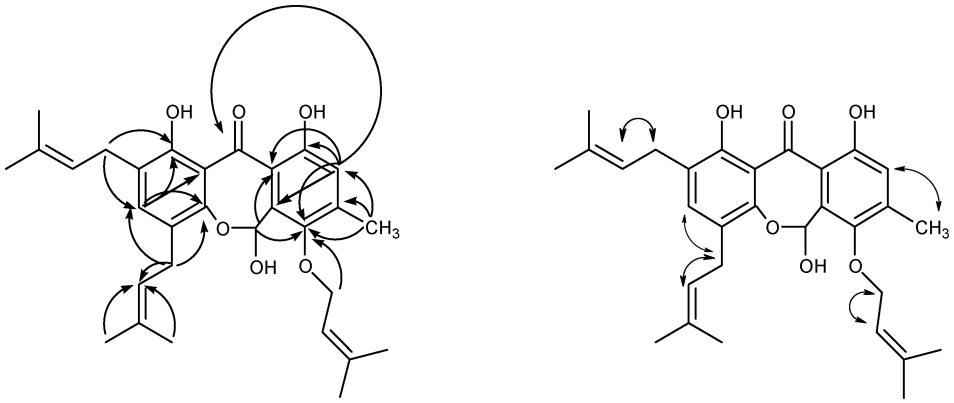


Figure 1. Significant HMBC (left, from H to C) and $^{1}H-^{1}H$ COSY (right) correlations for arugosin G (**1**).

major metabolites of *Aspergillus rugulosus*,[3a] *A. variecolor*,[5b] and *A. silvaticus*.[3b] Arugosins C,[6] D,[5b] and E[3b] also occur in *Aspergillus* spp., whereas arugosin F was found in *Ascodesmis sphaerospora*.[4] Biosynthetic studies[5,11] suggested that the bi- and tricyclic arugosins and compounds from the xanthone family are biogenetically related. Arugosin H (**2**) may be derived from chrysophanol anthrone, which undergoes oxidative cleavage to form the aldehyde function, followed by *C*-prenylation and hydroxylation (see Scheme 1). The aldehyde function can be converted to a hemiacetal function, as seen in the further prenylated and tricyclic arugosins G, A, and B (**1**, **3**, and **4**). Alternatively, cyclodehydration of the benzophenone intermediate yields shamixanthone (**5**) and emericellin (**6**).

Compounds **1**−**9** were tested in antibacterial, antifungal, and antialgal assays[12] at the 50 μg/disk level. Compound **2** showed inhibition zones against *Mycotypha microspora* (3 mm) and *Chlorella fusca* (2 mm); compounds **3** and **4** (as a mixture) were active against *Bacillus megaterium* (4 mm), while compound **9** inhibited *M. microspora* (11 mm) and *C. fusca* (5 mm).

The effects of the crude extract as well as the pure compounds **1**−**8** on tumor growth in vitro were investigated in a survival and proliferation assay using a panel of 36 human tumor cell lines representing 11 different tumor types. Antitumor activity was defined as test/control value smaller than 50% compared to the untreated control cells. The crude extract effected antitumor activity in all 36 cell lines (100%) at 50 μg/mL, in 31 out of the 36 cell lines (86%) at 5 μg/mL, and in 2/36 (6%) cell lines at 0.5 μg/mL. This is indicative of a selective and concentration-dependent antitumor activity of this extract and one or more of its ingredients. Among the pure compounds, compound **7** gave the highest activity score, exhibiting a mean IC_{50} value of 5.5 μg/mL. At a concentration of 10 μg/mL, 33 out of 36 cell lines (92%) were inhibited. As expected, the reference compound adriamycin tested in parallel in the same assays was more potent (IC_{50} 0.016 μg/mL). Compounds **3** and **4** were active in 7 out of the 36 cell lines (19%) at the highest concentration of 10 μg/mL. The other five compounds showed either only marginal or no antitumor activity in vitro.

Table 2. 1D and 2D NMR Spectral Data for Compound 2

atom no.	$^{13}C^{a,b}$ (δ in ppm)	$^{1}H^{a,b}$ (δ ppm, mult., J in Hz)	$^{1}H-^{1}H$ COSY^{a,b}	HMBC^{a,c}
1	162.0 (C)			
2	121.0 (C)			
3	138.4 (CH)	7.19 (d, 8.20)	1′,[d] 4	1, 1′, 4a,
4	106.8 (CH)	6.29 (d, 8.20)		2, 4a,[d] 9,[d] 9a
4a	159.4 (C)			
5	127.5 (CH)	7.04 (s)	6-Me	7, 8a, 9,[d] 10a[d]
6	128.6 (C)			
6-Me	15.3 (CH_3)	2.07 (s)	5	5, 7, 8
7	154.5 (C)			
8	117.6 (C)			
8a	131.7 (C)			
9	200.4 (C)			
9a	113.0 (C)			
10a	146.0 (C)			
11	196.2 (CH)	9.86 (s)		
1′	27.8 (CH_2)	3.24 (d, 7.32)	4′, 5′ 2′, 3	1, 2, 3, 2′, 3′, 4′,[d] 5′[d]
2′	123.1 (CH)	5.29 (t, 7.32)	1′, 4′, 5′	1′, 4′, 5′
3′	132.8 (C)			
4′	25.9 (CH_3)	1.69 (s)		3′, 5′
5′	17.8 (CH_3)	1.69 (s)		3′, 4′
OH		12.84 (s)		
OH		11.30 (s)		

[a] Acetone-d_6, 300/75.5 MHz. [b] Assignments are based on extensive 1D and 2D NMR measurements (HMBC, HSQC, COSY). [c] Numbers refer to carbon resonances. [d] Weak signal.

Compound **9** is known from the literature[13] to be a potent cytotoxic agent and was thus not evaluated in the current study.

The immunostimulating effects of compounds **1**−**8** were investigated by analyzing the stimulation of cytokine production by PBMCs from two healthy donors. All compounds were tested at concentrations of 0.1 and 1 μg/mL. These concentrations were not cytotoxic in the monolayer cytotoxicity and proliferation assay. LPS at 1 μg/mL, PMA at 10 ng/mL, and ionomycin at 1 μg/mL were used as positive controls. Twenty-four hours after exposure of PBMCs to the test compounds, PBMC supernatants were quantitatively tested for IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ by flow cytometry with the cytometric bead array (CBA).[14] None of the compounds induced the production of any of the cytokines. Negative PI-staining of PBMCs after removal of the supernatant confirmed that the failure of the test compounds to induce cytokine production by PBMCs was not due to cytotoxicity.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Jasco DIP 140 polarimeter. UV and IR spectra were obtained using Perkin-Elmer Lambda 40 and Perkin-Elmer Spectrum BX instruments, respectively. All NMR spectra were recorded on a Bruker Avance 300 DPX spectrometer. Spectra were referenced to residual solvent signals with resonances at $\delta_{H/C}$ 2.04/29.8, ($[CD_3]_2CO$) and 7.26/77.0 ($CDCl_3$). HREIMS were recorded on a Finnigan MAT 95 spectrometer. HPLC was carried out using a Merck-Hitachi system consisting of a L-6200A pump, a L-4500 photodiode array detector, and a D-6000 interface, or a Waters system with a 515 HPLC pump and a Knauer K-2300 differential refractometer as detector.

Isolation and Taxonomy of the Fungal Strain. Algal material was collected from Sardinia (Italy, Mediterranean Sea). After surface sterilization with 70% ethanol, algal samples were rinsed with sterile water and pressed onto biomalt agar plates to detect the presence of any fungal spores on the surface of algae. Sterilized algae were then cut into pieces and placed on agar plates containing isolation medium: 15 g/L agar, artificial seawater, benzyl penicillin (250 mg/L), and streptomycin sulfate (250 mg/L). Fungal colonies growing out of the algal tissue were transferred to medium for sporulation (15 g/L agar, 20 g/L biomalt extract, artificial seawater). The fungal strain was identified as *Emericella nidulans* var. *acristata* (Fennell & Raper) Subramanian by the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

Cultivation. The fungal strain (strain number Sar 14 15E, culture collection of Institute for Pharmaceutical Biology, University of Bonn, Germany) was cultivated at room temperature for one month in 32 × 8 L Fernbach flasks. The solid biomalt medium contained 20 g/L of Biomalt (Villa Natura Gesundheitsprodukte GmbH, Germany), 17 g/L agar (Fluka Chemie AG), and artificial seawater [(g/L): KBr (0.1), NaCl (23.48), $MgCl_2 \times H_2O$ (10.61), $CaCl_2 \times 2H_2O$ (1.47), KCl (0.66), $SrCl_2 \times 6H_2O$ (0.04), Na_2SO_4 (3.92), $NaHCO_3$ (0.19), H_3BO_3 (0.03)].[15]

Extraction and Isolation. The fungal biomass, including the medium, was homogenized using an Ultra-Turrax, and the mixture was extracted with EtOAc (3 × 8 L). After evaporation of the organic phase, 47 g of dark purple oil was obtained. The extract was fractionated by VLC (Si gel 60, 0.063−0.200 mm, Merck), with a CH_2Cl_2/EtOAc/MeOH gradient, to yield 11 fractions. Of these, fractions 2−6, on the basis of TLC results, were combined and separated on a Sephadex LH-20 (Pharmacia Biotech) column, with MeOH as eluent, to give five fractions (1−5). Sephadex fraction 2 was further separated on a normal-phase HPLC column (Knauer Si Eurospher-100, 250 × 8 mm, 5 μm), eluting with petroleum ether/acetone (9:1), and yielded 10 fractions (S1−S10). Of these, fraction S3 was identified as a mixture of compounds **3** and **4** (80.3 mg), whereas fraction S7 contained compound **7** (8.5 mg) and fraction 10 compound **2** (10.8 mg). Fraction S1 was separated into compounds **5** (4.7 mg) and **6** (5.1 mg) with *n*-hexane/EtOAc (20:1) using normal-phase HPLC (Knauer Si Eurospher-100, 250 × 8 mm, 5 μm). Fraction S2 was eluted on reversed-phase HPLC (Phenomenex Max C_{12}, 250 × 4.6 mm, 5 μm) with MeOH/H_2O (9:1) and yielded compound **1** (2.8 mg). Sephadex fraction 5 gave compound **8** (1.5 mg) after fractionation on reversed-phase (Knauer, Eurospher-100, C-8, 250 × 8 mm, 5 μm) HPLC with MeOH/H_2O (8:2). Sephadex fraction 3 was further fractionated using reversed-phase HPLC (Knauer C_8 Eurospher-100, 250 × 8 mm, 5 μm) with gradient elution from MeOH/H_2O (7:3) to MeOH in 90 min, 1.5 mL/min, to afford compound **9** (4.0 mg).

Arugosin G (1): bright yellow solid (2.8 mg); $[\alpha]^{24}_D$ −1.1 (*c* 0.29, MeOH); UV (MeOH) λ_{max} (log ϵ) 226 nm (sh) (4.20), 271 nm (3.84), 305 nm (3.82), 366 nm (3.75); IR (ATR) ν_{max} 3438, 2920, 1608, 1477, 1422, 1344, 1213, 1115, 1071, 998; ^1H and ^{13}C NMR data, see Table 1; HREIMS *m*/*z* 492.2517 (calcd for $C_{30}H_{36}O_6$ 492.2512).

Arugosin H (2): bright orange solid (10.8 mg); UV (MeOH) λ_{max} (log ϵ) 225 nm (4.50), 274 nm (4.33), 291 nm (sh) (4.21), 382 nm (3.93); IR (ATR) ν_{max} 3237, 2921, 1698, 1615, 1417, 1353, 1222, 1111, 1047, 982, 899, 816 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; HREIMS *m*/*z* 356.1266 (calcd for $C_{20}H_{20}O_6$ 356.1260).

Arugosin A (3) and B (4): 80.3 mg; NMR data identical with those previously published.[3b,6]

Shamixanthone (5): 4.7 mg; $[\alpha]^{24}_D$ +10.0 (*c* 0.51, $CHCl_3$) (lit. +11.9 (*c* 1.92, $CHCl_3$);[11] +25.2 (*c* 0.33, $CHCl_3$));[16] NMR data identical with those previously published.[5a,11]

Emericellin (6): 5.1 mg; identified by comparison of the ^{1}H and ^{13}C NMR data with those previously published.[3b]

Emindole DA (7): 8.5 mg; $[\alpha]^{24}_D$ −23.4 (*c* 0.86, MeOH) (lit. −30.7 (*c* 2.32, MeOH));[7] NMR data identical with those previously published.[7]

Microperfuranone (8): 1.5 mg; $[\alpha]^{24}_D$ −4.4 (*c* 0.27, MeOH) (lit. −6.8 (*c* 0.60, MeOH));[8] NMR data identical with those previously published.[8]

Sterigmatocystin (9): (4.0 mg); $[\alpha]^{24}_D$ −106.2 (*c* 0.25, $CHCl_3$); identified by comparison of the ^{1}H and ^{13}C NMR data with those previously published.[9]

Bioassays. Antimicrobial Activity. Activities of compounds **1**−**9** were tested in agar diffusion assays against the bacteria *Bacillus megaterium* and *Escherichia coli*, the fungi *Microbotryum violaceum*, *Eurotium repens*, and *Mycotypha microspora*, and the green microalga *Chlorella fusca*. The tests were performed as previously reported.[12]

Antitumor Activity. Antitumor activity of the crude extract and compounds **1**−**8** was tested in a monolayer cytotoxicity and proliferation assay using human tumor cell lines as described previously.[17] Briefly, the number of viable cells after 4 days of incubation with a test compound was determined using propidium iodide fluorescence as a read-out. Antitumor activity including the induction of apoptosis and the inhibition of cell prolilferation was recorded as a reduction of the viable cell number relative to control wells and expressed as a T/C (test/control) value. The requirement of antitumor activity was a T/C value of ≤50%. The crude extract and the pure compounds **1**−**8** were tested in triplicate in a panel of 36 human tumor cell lines at five different concentrations ranging from 0.005 to 50 μg/mL (crude extract) and 0.001 to 10 μg/mL (compounds **1**−**8**), respectively. Twenty-four out of the 36 test cell lines had been established from patient-derived tumor xenografts growing in nude mice as described by Roth et al.[18] The origin of the donor xenografts was described by Fiebig et al.[19] The remaining 12 cell lines were kindly provided by the U.S. National Cancer Institute or purchased from the American Type Culture Collection (Rockville, MD).

Immunostimulating Activity. For the isolation of PBMCs, freshly drawn human blood was treated with EDTA as an anticoagulant. Cells were diluted with 3 volumes of CliniMACS PBS/EDTA buffer (Miltenyi, Bergisch Gladbach), carefully layered over FicollPaque (Amersham Biosciences, Freiburg) in a conical tube, and centrifuged at 400*g* for 40 min at 20 °C in a swinging-bucket rotor without brake. The upper layer was aspirated, leaving the mononuclear cell layer undisturbed at the interphase. The interphase cells (lymphocytes, monocytes, and thrombocytes) were carefully transferred into a new conical tube. The conical tube was filled with CliniMACS PBS/EDTA buffer and centrifuged at 300*g* for 10 min at 20 °C. The supernatant was removed completely. For removal of platelets the cell pellet was resuspended in 50 mL of buffer and centrifuged at 200*g* for 10 min at 20 °C. The supernatant was completely removed and the final washing step was repeated. Cells were resuspended in DMEM medium (Invitrogen, Karlsruhe) and counted in a Neubauer hemocytometer.

For the stimulation of PBMCs, 250 000 cells per well were seeded in a 96-well plate. PBMCs of two different healthy donors were stimulated with compounds **1**−**8**. For the stimulation of PBMCs two noncytotoxic concentrations (0.1 and 1 μg/mL), as determined before by the monolayer cytotoxicity and proliferation assay, were used for each compound. LPS at 1 μg/mL (Linaris, Wertheim-Bettingen) or PMA at 10 ng/mL (Sigma, Deisenhofen) and ionomycin at 1 μg/mL (Sigma, Deisenhofen) were used as positive controls. Cells were incubated at 37 °C and 5% CO_2 in a humidified atmosphere for 24 h.

The cytometric bead array (CBA) technology[14] allows for quantitative detection of up to seven cytokines in a single sample. The CBA employs different particles with discrete fluorescence intensities that are coated with distinct capture antibodies to detect cytokines in the supernatant. The capture beads are mixed with different phycoerythrin-conjugated detection antibodies for quantitative determination of the respective cytokine. Standard mixtures are used to generate standard curves for all the cytokines. The supernatants of the stimulated PBMCs were taken immediately prior to analysis for cytokines. The supernatants from PBMCs stimulated either with LPS, or PMA and ionomycin are used as internal positive controls for the CBA. The cytokines IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ were quantitatively measured with a Coulter Cytomics FC500 cytometer using the CBA (Becton Dickinson, San Diego) according to the manufacturer's intructions. The results were analyzed with the Coulter Cytomics Bead Array Analysis program.

After removal of the cytokine-containing supernatant, PBMCs were tested for viability by flow cytometry. Propidium iodide staining solution (0.1 μg/ test of 1×10^6 cells) was used to determine the amount of dead cells. Unstimulated PBMCs were used as a negative control. PBMCs that were frozen and thawed twice were used as a positive control.

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